

ICOS and B7 Costimulatory Molecule Expression Identifies Activated Cellular Subsets in Rheumatoid Arthritis

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To better define important cell subsets expressing activation markers in rheumatoid arthritis (RA), we compared selective lymphocyte and monocyte B7H1, B7H2, B7RP.1, B7RP.2, and inducible costimulatory molecule (ICOS) expression from normal peripheral blood (NL PB), RA PB, and RA synovial fluid (SF) by multicolor flow cytometry and immunohistochemistry. RA SF memory lymphocytes expressed B7RP.1 and B7RP.2, suggesting that T-cells may function as antigen presenting cells (APCs) in RA joints. We found similar results for ICOS expression. RA SF CD14+ monocytes also expressed B7RP.1 (an ICOS ligand) and the homologous ligand B7RP.2, identifying monocytes as potential mediators of antigen processing and lymphocyte activation in RA. Furthermore, we found an increased population of RA SF CD14+ monocytes expressing B7H1 and B7H2. [The FACS analysis was sup-

ported by immunohistochemistry, showing intense lymphocyte and APC (macrophages with dendritic morphology) ICOS staining in RA synovial tissue (ST). Overall, these results define elevated populations of memory T-lymphocytes expressing proinflammatory B7 molecules in RA SF that either stimulate T cells through ICOS (via ICOS ligands B7RP.1 and B7RP.2), or down-regulate RA ST T-lymphocytes through B7H1 and B7H2.] Therefore, in the same joint, there may exist positive and negative influences on the inflammatory response, and perhaps, the negative signals dominate as joint inflammation resolves.

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Key terms: rheumatoid arthritis; B7; ICOS; inflammation; antigen presentation

The inducible costimulatory molecule (ICOS) is a T-cell associated receptor involved in cell activation that is conservatively expressed on memory T-cell subsets (1). ICOS is a 60 kDa, disulfide-linked, glycosylated homodimer (1) expressed on T-lymphocytes in lymphoid organs, such as spleen, lymph node, and Peyer's patches in both humans and mice (1–5). ICOS expressing T cells are also found in germinal centers that are enhanced after immune priming (1,5). ICOS performs several functions in immune activation including binding B7 accessory molecules on antigen presenting cells (APCs). This binding results in activation of responding lymphocytes (1,3). Because they activate T-cells, ICOS and T-lymphocyte associated molecules, CTLA4 and CD28, have been designated as potential therapeutic targets to treat inflammatory diseases. Akin to ICOS, CD28 and CTLA4 are quite unique in that these

molecules better define immune function. For example, B7 ligands found on dendritic cells (and on B-lymphocytes and monocytes) bind to both T-cell associated CD28 (activating) and CTLA4 (inhibitory) cell receptors. After engagement of antigen and accessory molecules by the T-cell receptor (TCR), B7 ligands will direct T-cell responses (1). Therefore, cell-mediated immunity continues unimpeded or is attenuated depending upon which TCR is bound.

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Interestingly, expression of ICOS on T lymphocytes depends upon TCR and CD28 signaling mechanisms. Activation of T cells without CD28 engagement results in diminished levels of T-cell ICOS expression (1,6). Although complex, this elegant system ensures that immune responses are turned on or off appropriately. This process has been demonstrated in vivo in a murine model of experimental allergic encephalomyelitis (EAE) in which it was shown that ICOS protein and mRNA levels were upregulated on infiltrating CD3⁺ T-cells directly before disease onset (7). ICOS blockade during the efferent immune response (9–20 days after immunization) abrogated EAE development (7). This finding was supported in murine collagen induced arthritis (CIA) in which blocking B7 and ICOS interactions significantly ameliorated joint inflammation (8). It was also reported that ICOS gene deficient mice bred on the CIA susceptible DBA-1 background were completely resistant to CIA development, joint inflammation, and proinflammatory cytokine expression (9).

The data presented in this study extend and clarify the expression of ICOS and B7 costimulatory molecules in rheumatoid arthritis (RA). We define the expression of ICOS, B7H1, B7H2, B7RP.1, and B7RP.2 on CD14⁺ monocytes, and on naive (CD45RA⁺) and memory (CD45RO⁺) lymphocytes from normal (NL) peripheral blood (PB), RA PB, and RA synovial fluid (SF) by flow cytometry. We further show by immunohistochemistry that the ligands for ICOS, namely B7RP.1 and its homologous ligand B7RP.2, are prominently expressed on RA synovial tissue (ST) macrophages, whereas RA ST lymphocytes express ICOS. This data indicates that lymphocyte ICOS expression, and the macrophage associated ligands B7RP.1 and B7RP.2 are expressed in RA, and may be responsible, at least in part, for the progressive inflammatory responses observed in the RA joint.

MATERIALS AND METHODS

Patient Samples

SF samples were obtained during arthrocentesis from patients with RA, and PB was collected in heparinized tubes from patients with RA or from healthy NL volunteers. STs from RA patients were obtained from patients undergoing total joint replacement who met the American College of Rheumatology criteria for RA.

Flow Cytometry

SF cells were collected by passing RA SF through a 40 μ m nylon mesh filter. Filtered SF was centrifuged, leaving a cell pellet. RA SF cells were washed 3 \times in FACS buffer [PBS/1% fetal bovine serum (FBS)] and resuspended at a concentration of 1×10^7 cells/ml in blocking buffer (1% BSA/0.1% NaN₃/30% goat serum in PBS) for 15 min at 4°C. SF cells (1×10^6 per tube) or 100 ml of whole blood were incubated with primary antibodies directed against ICOS, B7H1, B7H2, B7RP.1, and B7RP.2 or appropriate IgG isotype controls (Sigma, St. Louis, MO) at 5 mg/ml in blocking buffer for 30 min at 4°C. Cells were washed with

FACS buffer, then incubated with goat anti-mouse IgG R-phycoerythrin (PE; Jackson Immunoresearch Laboratory, West Grove, PA) diluted 1:100 in blocking buffer for 30 min at 4°C. Cells were washed and incubated with 20 μ l mouse serum for 10 min at room temperature (RT). Cychrome-conjugated CD3 (detects T cells) mAbs (PharMingen, San Diego, CA) were added for 30 min at 4°C.

We evaluated ICOS or B7 costimulatory receptor family expression on naive or memory leukocytes by incubating cells with either FITC-labeled mouse anti-human CD45RA (PharMingen, San Diego, CA), which detects naive leukocytes, or mouse anti-human CD45RO (PharMingen, San Diego, CA), which detects memory leukocytes. SF cells were washed 2 \times in FACS buffer and fixed in 500 μ l of fresh 1% formaldehyde in PBS. Whole blood RBCs were lysed with Becton-Dickinson Lysing Reagent (Becton-Dickinson, Mountain View, CA) before fixation. ICOS and B7 expression on lymphocytes and monocytes was determined by first gating on cells using side scatter properties. Receptor expression on cells was determined by a second histogram of the gated CD3⁺ lymphocytes (FITC) or CD14⁺ monocytes (FITC) against ICOS or B7 molecule expression (PE) fluorescence. For some studies, chemokine receptor expression on lymphocyte subsets was evaluated by incubating cells with anti-CD4-ECD or anti-CD8-ECD labeled antibodies (Beckman-Coulter, Miami, FL), added along with FITC (anti-CD45RA or anti-CD45RO) and cychrome (anti-CD3, PharMingen, San Diego, CA) labeled antibodies, using additional histograms to evaluate memory or naive CD3⁺/CD4⁺ and CD3⁺/CD8⁺ positive lymphocytes. Cells were analyzed using a Coulter Epics XL flow cytometer (Coulter, Hialeah, FL) and the percentages of positive cells determined. The numbers of cell analysis counts were routinely between 75,000 and 200,000 events per tube, depending on the experiment, with $\sim 1 \times 10^6$ cells per tube for all experiments. Percentages of cells expressing ICOS or a particular B7 costimulatory molecule were calculated after defining a background value for the isotype matched control antibody.

Immunohistochemistry

Frozen RA ST sections (7 μ m) were cut and immunoperoxidase stained with an avidin-biotin technique (Vector Laboratories, Burlingame, CA) with all subsequent incubations being performed at 37°C in a humidified chamber. Slides were fixed in cold acetone for 20 min, then treated with 3% peroxidase in 0.1 M Tris for 5 min to block endogenous peroxidase activity. Tissues were blocked with 3% horse serum (in PBS) for 1 h, then incubated with mouse 10 μ g/ml anti-human antibody (Millennium Pharmaceuticals, Cambridge, MA), or purified nonspecific mouse antibody negative control (Coulter, Miami, FL) for an additional hour. Antibodies against ICOS, B7H1, B7H2, B7RP.1, and B7RP.2 (Millennium Pharmaceuticals, Cambridge, MA) were made as previously described (10). Tissue was washed twice in PBS, and a 1:200 dilution (in PBS) of anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, MA) was added to the tissue sections and incubated

for an additional 20 min. After a final washing (2× in PBS), slides were developed with a diaminobenzidine tetrahydrochloride substrate (Vector Laboratories) for 2 min at RT, rinsed in tap water, counter-stained with Harris' Hematoxylin, and dipped in saturated lithium carbonate solution for bluing.

[Serial tissue sections were examined by a blinded pathologist to determine the percentage of each cell type expressing B7 molecules in NL and RA ST. Various ST cell types were identified including macrophages, lymphocytes, fibroblasts, endothelial cells, and APCs with dendritic morphology (referred to as dendritic-like cells) by immunohistochemical staining reaction and/or morphological features. Human macrophages were identified morphologically and with anti-LeuM5 (Becton Dickinson, San Jose, CA) and were CD68+ (CD68 stain is clone EBM-11, Dako, IgG1, Carpinteria, CA) in serial sections. Endothelium was verified using anti-von Willebrand's factor (Dako, Carpinteria, CA). Dendritic-like cells were identified with a dendritic cell marker (OX-62 IgG, PharMingen, San Diego, CA) as previously described (11). Immunostaining was graded by a frequency of staining scale (0–100%), where 0% indicated no staining and 100% showed that all the cells were immunoreactive for each of the ST components.]

Statistics

PB and SF samples were analyzed by the Student's independent *t* test. Comparisons of values where *P* < 0.05 were considered to indicate statistical significance. Error bars on graphs represent the standard error of the mean.

RESULTS

Flow Cytometry Analysis of CD3+ Lymphocytes in NL PB, RA PB, and RA SF

Two color FACS analysis of CD3+ lymphocyte populations obtained from NL PB, RA PB, and RA SF for ICOS and B7 molecule expression is shown in Figure 1. FACS analysis revealed a significant CD3+ lymphocyte population in RA SF expressing B7RP.1 and B7RP.2 compared to lymphocyte populations in NL or RA PB (Fig. 1).

Four-Color Flow Cytometry Analysis of Naive CD45RA+/CD3+/CD4+ and CD45RA+/CD3+/CD8+ Lymphocyte Subsets in NL PB, RA PB, and RA SF

We performed FACS analysis of ICOS, B7RP.1, B7RP.2, B7H1, and B7H2 expression on naive CD45RA+/CD3+/CD4+ and CD45RA+/CD3+/CD8+ lymphocytes from NL PB, RA PB, and RASF. This is highlighted in Figure 2, where it is clear that naive CD45RA+/CD3+/CD4+ lymphocytes did not significantly express either ICOS or B7 molecules in any of the patient fluids examined (Fig. 2A, upper panel), with groups showing the percentage of expression under 1%. Figure 2B (lower panel) shows the FACS analysis for CD45RA+/CD3+/CD8+ lymphocytes in which expression of ICOS and B7 costimulatory mole-

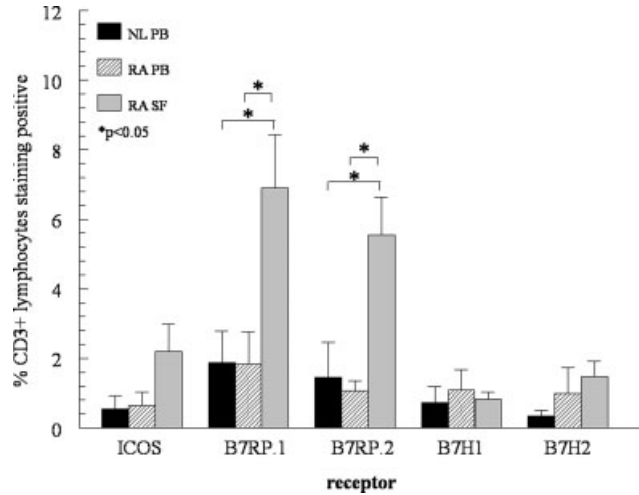


Fig. 1. Percentages of CD3+ lymphocytes from PB (ICOS *n* = 4; B7RP.1 *n* = 4; B7RP.2 *n* = 4; B7H1 *n* = 4; B7H2 *n* = 4), RA PB (ICOS *n* = 5; B7RP.1 *n* = 4; B7RP.2 *n* = 4; B7H1 *n* = 4; B7H2 *n* = 4), and RA SF (ICOS *n* = 8; B7RP.1 *n* = 7; B7RP.2 *n* = 7; B7H1 *n* = 7; B7H2 *n* = 6). Note the increased populations of B7RP.1 and B7RP.2 expressing CD3+ lymphocytes in RA SF compared to NL and RA PB (*n*, number of different patients).

cules were all relatively low for all groups, lacking statistical significance for ICOS and B7 molecules examined between the NL PB, RA PB, and RA SF groups. The lack of ICOS and B7 molecule expression on CD3+/CD4+/CD45RA+ and CD3+/CD8+/CD45RA+ lymphocytes is not surprising since we typically do not find large populations of naive lymphocytes in RA SF on whom inflammatory markers such as chemokine receptors are usually expressed (10).

[It should be noted that the percentages of naive T-cells, including CD3/CD4 and CD3/CD8 are low in the RA SF, but higher in the RA and NL PB. Specifically, T-cells carrying the CD45RA+ or CD45RO+ phenotypes are ~30–40% in the NL and RA PB, however, only 10% of naive T-cells compared to 40% of activated T-cells are in the RA SF (10). This is presumably a result of the inflammatory environment of the RA joint activating local T-cell populations.]

Four-Color Flow Cytometry Analysis of Memory CD45RO+/CD3+/CD4+ and CD45RO+/CD3+/CD8+ Lymphocyte Subsets in NL PB, RA PB, and RA SF

Figure 3A (upper panel) shows the overall results of memory lymphocyte CD45RO+/CD3+/CD4+ ICOS, B7RP.1, B7RP.2, B7H1, and B7H2 expression from NL PB, RA PB, and RA SF. As shown, the percentages of B7RP.1 and B7RP.2 expressing lymphocytes were significantly elevated in RA SF compared to NL PB and RA PB; whereas no significant differences were found in the groups expressing B7H1 or B7H2.

Figure 3B shows the population of memory CD45RO+/CD3+/CD8+ ICOS, B7RP.1, B7RP.2, B7H1, and B7H2

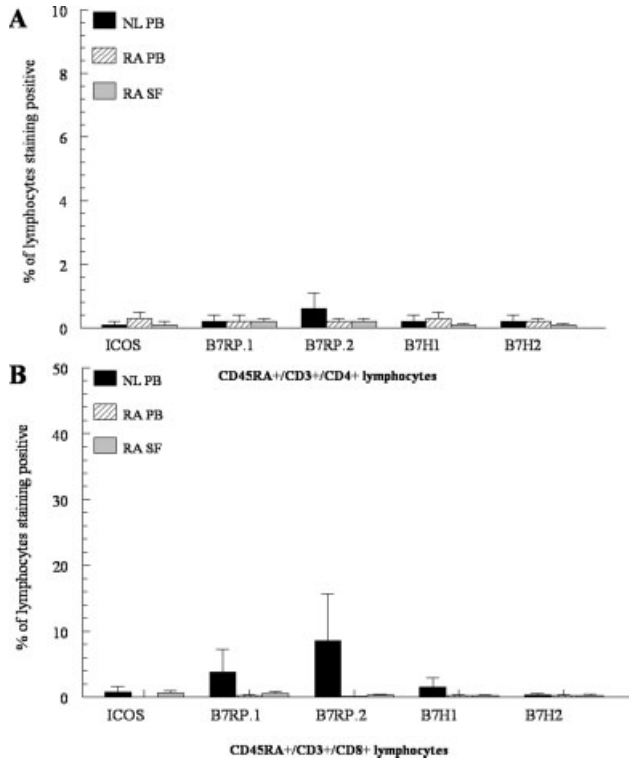


FIG. 2. Comparisons of the number of naïve CD45RA+/CD3+/CD4+ lymphocytes from NL PB (ICOS $n = 4$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), RA PB (ICOS $n = 5$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), and RA SF (ICOS $n = 8$; B7RP.1 $n = 7$; B7RP.2 $n = 7$; B7H1 $n = 7$; B7H2 $n = 7$; upper panel) and CD45RA+/CD3+/CD8+ lymphocytes from NL PB (ICOS $n = 4$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), RA PB (ICOS $n = 5$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), and RA SF (ICOS $n = 8$; B7RP.1 $n = 7$; B7RP.2 $n = 7$; B7H1 $n = 7$; B7H2 $n = 7$; lower panel) is shown. Populations of naïve CD3+/CD4+ or CD3+/CD8+ lymphocytes expressing either ICOS or B7 molecules were only slightly above background for all groups in NL PB, RA PB, and RA SF (n , number of different patients).

expressing lymphocytes from NL PB, RA PB, and RA SF. No significant statistical differences were seen with CD45RO+/CD3+/CD8+ and any of the other groups examined. Figure 4 is a representative dot-plot of CD45RO+/CD3+/CD4+ lymphocytes from the various patient SFs. As shown in the dot-plots, large memory CD3+/CD4+ lymphocyte populations from the RA SF expressed both ICOS and the ICOS ligands B7RP.1 and B7RP.2.

Flow Cytometry Analysis of Memory CD14 Monocytes in NL PB, RA PB, and RA SF

CD14 monocyte expression of ICOS, B7RP.1, B7RP.2, B7H1, and B7H2 was analyzed by FACS analysis and the results are shown in Figures 5 and 6. As shown in Figure 5, an increased population of CD14+ monocytes expressing ICOS, B7RP.1, B7RP.2, B7H1, and B7H2 is seen in RA SF compared to both NL and RA PB. Figure 6 is a representative dot-plot showing the significantly elevated populations of RA SF CD14+ monocytes expressing B7 mole-

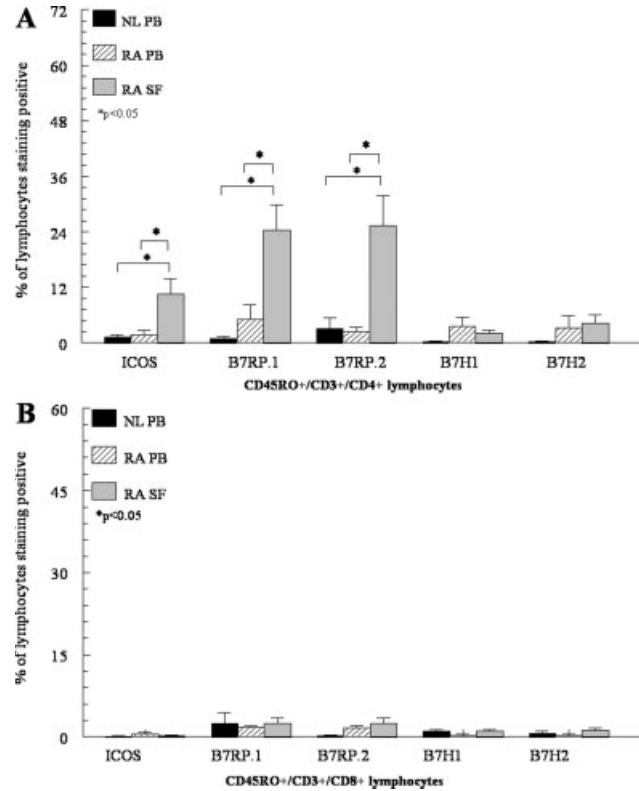


FIG. 3. Comparisons of the number of memory CD45RO+/CD3+/CD4+ lymphocytes from NL PB (ICOS $n = 4$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), RA PB (ICOS $n = 5$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), and RA SF (ICOS $n = 6$; B7RP.1 $n = 7$; B7RP.2 $n = 7$; B7H1 $n = 7$; B7H2 $n = 7$; upper panel) and CD45RO+/CD3+/CD8+ lymphocytes from NL PB (ICOS $n = 4$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), RA PB (ICOS $n = 5$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 4$; B7H2 $n = 4$), and RA SF (ICOS $n = 8$; B7RP.1 $n = 7$; B7RP.2 $n = 7$; B7H1 $n = 7$; B7H2 $n = 7$; lower panel) is shown. Populations of memory CD3+/CD4+, but not CD3+/CD8+ lymphocytes expressing ICOS and the ICOS ligands B7RP.1 and B7RP.2 molecules were elevated in RA SF compared to NL and RA PB ($P < 0.05$, n , number of different patients). Thus, RA SF CD3+ lymphocyte populations expressing B7RP.1 and B7RP.2 molecules in Figure 1 were likely memory CD45RO+/CD3+/CD4+ lymphocytes. This indicates that infiltrating memory lymphocytes may be induced to express antigen presenting accessory molecules and that these lymphocyte populations may participate in antigen processing in the RA joint.

cules as well as ICOS. Thus, APCs express B7 molecules and ICOS in RA SF, but PB CD14+ monocytes do not.

Immunohistochemistry of RA ST for ICOS and B7H2 Expression

[Figure 7 shows a photomicrograph of RA ST immunostained for ICOS. As indicated (see arrows), lymphocytes are immunoreactive for ICOS. As seen in Figure 8, RA ST dendritic-like cells were also intensely immunoreactive for B7H2. Immunoreactive leukocytes were blindly identified by morphological features by a pathologist (magnification 400 \times). Table 1 outlines cellular expression of B7H1, B7H2, B7RP.1, and B7RP.2 in RA and NL ST. As indicated, only B7H2 dendritic-like cell expression was significantly elevated in RA ST compared to NL ST.]

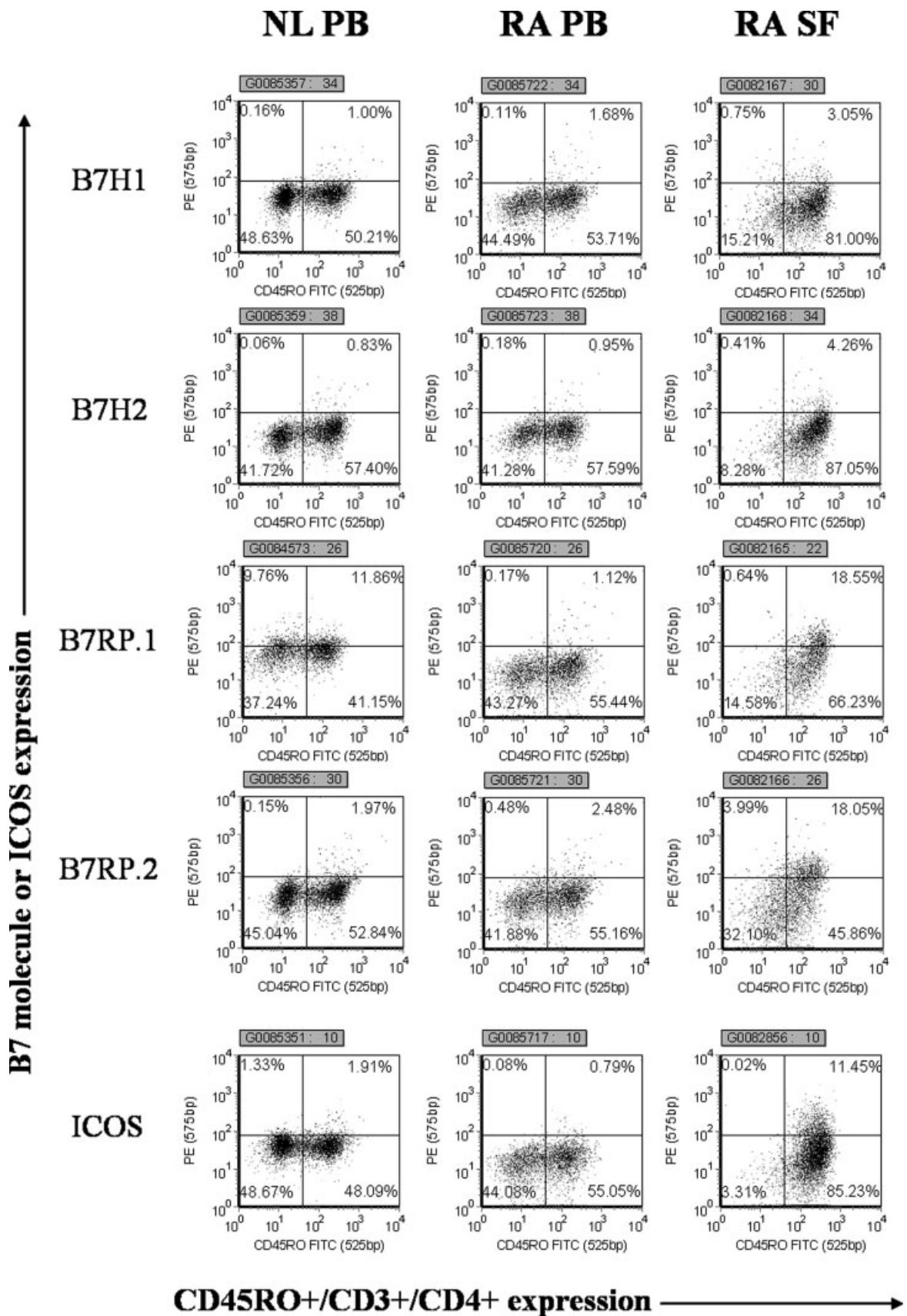


FIG. 4. Representative histograms (dot plots) showing ICOS, B7H1, B7H2, B7RP.1, and B7RP.2 expression on memory CD45RO+/CD3+/CD4+ lymphocytes. As shown, significant populations of memory CD3+/CD4+ lymphocytes expressing ICOS, and the ICOS ligands B7RP.1, and B7RP.2 were found in the RA SF, but not NL or RA PB. NL, normal; PB, peripheral blood; SF, synovial fluid.

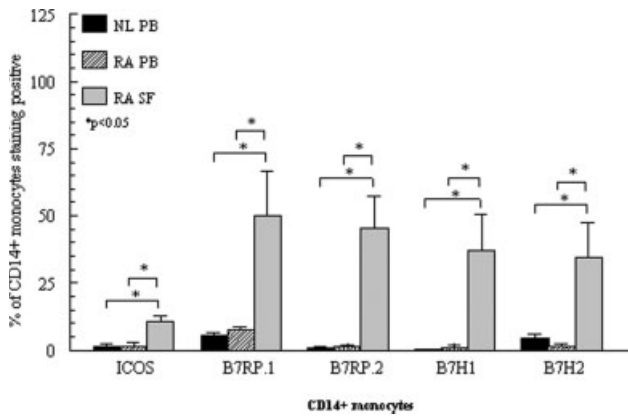


FIG. 5. Expression of ICOS and B7 accessory molecules on CD14⁺ monocytes from NL PB (ICOS $n = 5$; B7RP.1 $n = 5$; B7RP.2 $n = 5$; B7H1 $n = 5$; B7H2 $n = 4$), RA PB (ICOS $n = 4$; B7RP.1 $n = 5$; B7RP.2 $n = 5$; B7H1 $n = 5$; B7H2 $n = 5$), and RA SF (ICOS $n = 4$; B7RP.1 $n = 4$; B7RP.2 $n = 4$; B7H1 $n = 3$; B7H2 $n = 3$). Elevated populations of CD14⁺ monocytes expressing ICOS, the ICOS ligands B7RP.1, B7RP.2, and the PD-1 and PD-2 ligands B7H1 and B7H2 respectively were found in RA SF compared to NL or RA PB ($P < 0.05$, n , number of different patients). This indicates that monocytes in the RA joint respond to both positive and negative influences as joint inflammation progresses.

DISCUSSION

RA is an autoimmune disease of unknown etiology associated with increased angiogenic activity (12–14), and cellular recruitment to the joints (10,11,15,16). Inflammatory responses in the RA joint can be attributed in large part to activated T-cells in the RA ST and SF (17). T-cells become further activated via their constitutively expressed CD28 receptors when presented with antigen from an APC, along with appropriate signals from APC accessory molecules like B7.1 and B7.2. These interactions are known to provide potent proinflammatory signals to T-cells, resulting in autocrine IL-2 production, which is important for T-lymphocyte proliferation (1,3,5,18–21). Importance of CD28 costimulation has been directly demonstrated by blocking CD28 and B7 interactions and preventing T-cell responses in autoimmune disease models. In some cases, this has resulted in prolonged tissue acceptance in organ transplant models (1,22).

The newest members of the B7 family, namely B7H1, B7H2, B7RP.1, and B7RP.2, have several distinctive features including expression on B lymphocytes (23), and broader expression in nonlymphoid tissues (24). However, B7 molecule family members have also been shown to serve contrasting functions in immunology. For example, B7H1 and B7H2, also known as PD-L1 (program death ligand 1) and PD-L2, respectively, are known to be expressed on B cells and dendritic cells. They are also expressed on monocytes that deliver key lymphocytic apoptotic signals by engaging the PD-1 receptor on activated T-lymphocytes, thus attenuating certain immune responses (1). Conversely, ICOS is an activation marker expressed on activated T-cells that binds B7RP.1 expressing monocytes. ICOS engagement with B7RP.1, along with an appropriate antigen, is believed to induce critical activation signals to the T-cell that exacerbate the

response (1). This process implicates the antigen, along with an appropriate APC signal, to dictate how activated T-cells will respond. This produces a biased Type 1 (Th1) or Type 2 (Th2) cytokine mediated response that triggers associated chemokine secretion and selective cell recruitment to inflammatory sites (25).

Interestingly, ICOS engagement also augments induction of both Type 1 and Type 2 cytokines, indicating its importance in both polarized Type 1 and Type 2 inflammatory reactions. Correspondingly, Type 1 and Type 2 cytokines can also influence ICOS expression on numerous cell subsets. For example, ICOS expression is upregulated on murine NK cells, where ICOS dependent cytotoxicity and IFN- γ production is observed through the phosphoinositide 3-kinase pathway (26). Furthermore, in a murine model of airway hyperactivity, bronchial lymph node cell production of IL-10, a Type 2 associated cytokine, is mediated through the ICOS pathway (27). These studies indicate a strong relationship between ICOS expression and Type 1 and Type 2 immunity. However, the latter finding is further supported by a recent report showing intraperitoneal treatment of mice with anti-ICOS neutralizing antibodies during the sensitization phase with ovalbumin results in decreased airway eosinophilia and IL-5 production (28). Additionally, it has been shown that type 2 lymphocytes secreting IL-10 have elevated ICOS expression (29). Thus, Type 1 and Type 2 lymphocytes express ICOS, but it is generally acknowledged that Type 2 lymphocytes express greater amounts of ICOS than Type 1 cells (1,6,20,21,30). This could explain, in part, the attenuating effects of blocking ICOS during Type 2 inflammatory reactions. These findings strongly support the idea that ICOS blockade is a viable therapeutic strategy for targeting anamnestic, allergic type hypersensitivity reactions.

Because of the noticeably elevated expression of ICOS on Type 2 cells, it has been questioned whether ICOS blockade in Type 1 cytokine mediated reactions may show benefit in tempering Type 1 inflammation. This notion was recently tested in a Type 1 mediated murine EAE model in which ICOS blockade markedly reduced EAE severity (7). Specifically, EAE progression was significantly reduced by administering blocking antibodies to ICOS during the efferent phase of the immune response (days 9–20), a time when the disease course adopts a Type 1 profile. Interestingly, anti-ICOS antibody administration during the priming phase of EAE (days 0–9) exacerbated the disease, likely due to inhibition of an early Type 2 response. Development of this Type 2 reaction may have naturally tempered a later Type 1 response (7). In agreement with these findings is a report by Iwai et al., showing reduction of murine CIA development, a prototypical Type 1 disease, was achieved by administering neutralizing, anti-B7 antibodies capable of blocking B7 and ICOS interactions after the boosting (antigen priming) phase of CIA (8). In agreement, Nuriyeva et al., showed ICOS knockout CIA susceptible DBA-1 mice are completely resistant to CIA development and joint inflammation (9). Taken together, these results indicate ICOS and B7

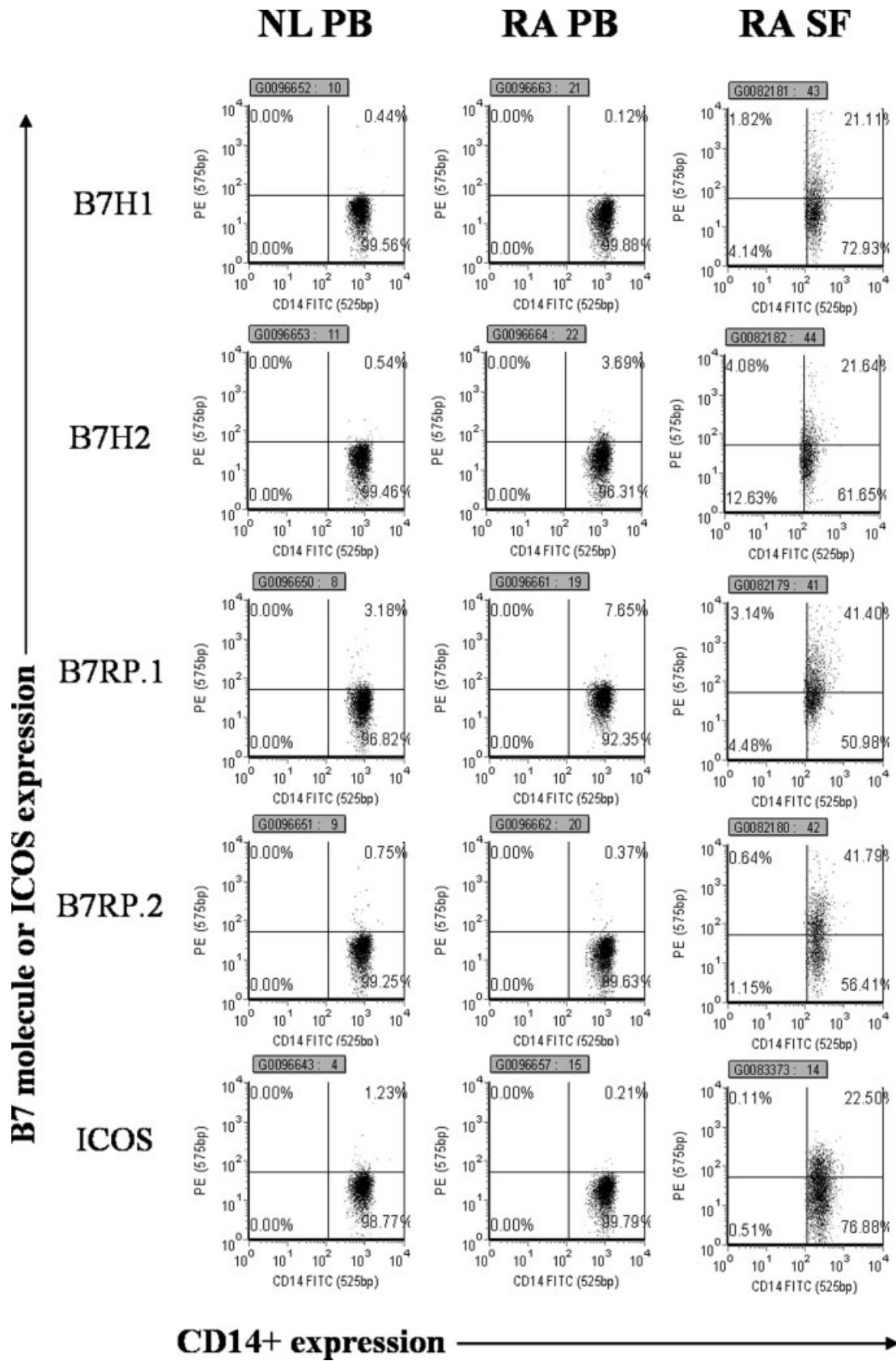


Fig. 6. Representative histograms (dot plots) showing ICOS, B7H1, B7H2, B7RP.1, and B7RP.2 expression on CD14+ monocytes from NL PB, RA PB, and RA SF. As shown, significant populations CD14+ monocytes expressing ICOS, and the ICOS ligands B7RP.1, and B7RP.2, as well as the PD-1 and PD-2 ligands B7H1 and B7H2 respectively were found in RA SF compared to NL and RA PB. NL, normal; PB, peripheral blood; SF, synovial fluid.

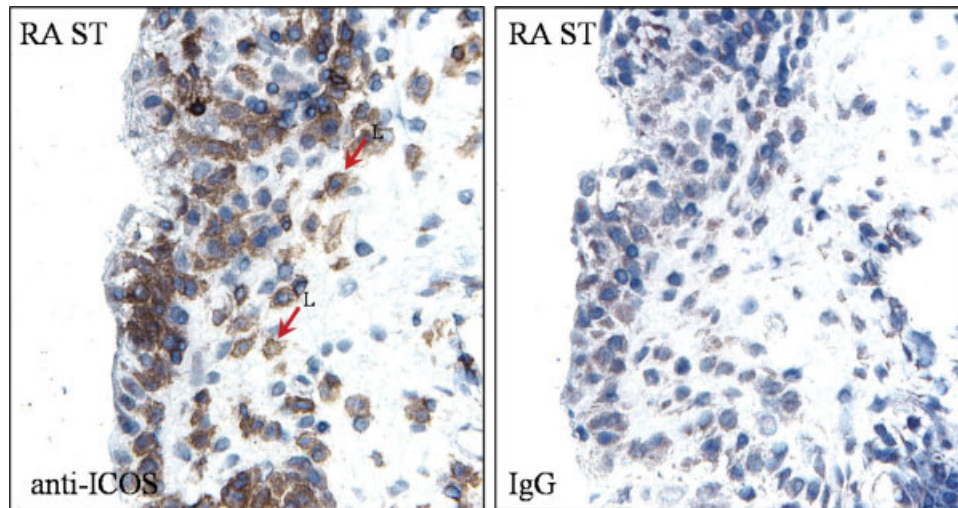


FIG. 7. Photomicrograph of RA ST immunostained for ICOS expression. As shown, scattered lymphocytes in the RA ST stained positive for ICOS (indicated as L, see arrows; original magnification $\times 400$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

molecules may play an integral part in the pathogenesis of CIA and other Type 1 diseases. These findings also indicate that antagonizing B7-ICOS interactions may be worthwhile in treating RA, assuming these molecules are significantly expressed in human RA tissues.

The recent study by Okamoto et al. describes ICOS expression on CD4⁺ RA SF cells, and shows by reverse transcriptase polymerase chain reaction (RT-PCR) that RA ST contained elevated mRNA levels of the ICOS ligand B7RP.1 (31). The data herein further defines upregulated populations of ICOS expressing cell subsets, including CD14⁺ monocytes in RA SF. Furthermore, we clearly demonstrate upregulation of B7 molecules on RA SF CD14⁺ monocytes, indicating activation of the innate immune response in RA patients. We also identified $\sim 25\%$ of RA SF memory CD3⁺/CD4⁺ lymphocytes expressing B7RP.1 or B7RP.2, and $\sim 10\%$ of memory CD3⁺/CD4⁺ lymphocytes expressing ICOS. Thus, lymphocytes, as well

as monocytes can express ICOS and B7 molecules in RA SF compared to NL or RA PB. This is consistent with our previous study showing T-cells express B7 molecules in the RA ST (17). It is tempting to speculate that lymphocytes and monocytes may swap ICOS and B7 molecules during antigen presentation in the RA joint, where there is abundant antigen. This exchange would explain the elevated expression of B7 that allows synovial T cells to serve as functional APCs in the rheumatoid joint (17). By this method, functional immune cells could increase or decrease reactivity in the RA joint via the ICOS and/or CD28 pathways.

With respect to ICOS expression on cell subsets during inflammatory responses, the question arises whether ICOS expressing leukocyte populations are specifically recruited to the RA joint, or stimulated to express ICOS upon entering the joint? The latter explanation is unlikely considering that *in vivo* interperitoneal blockade of ICOS

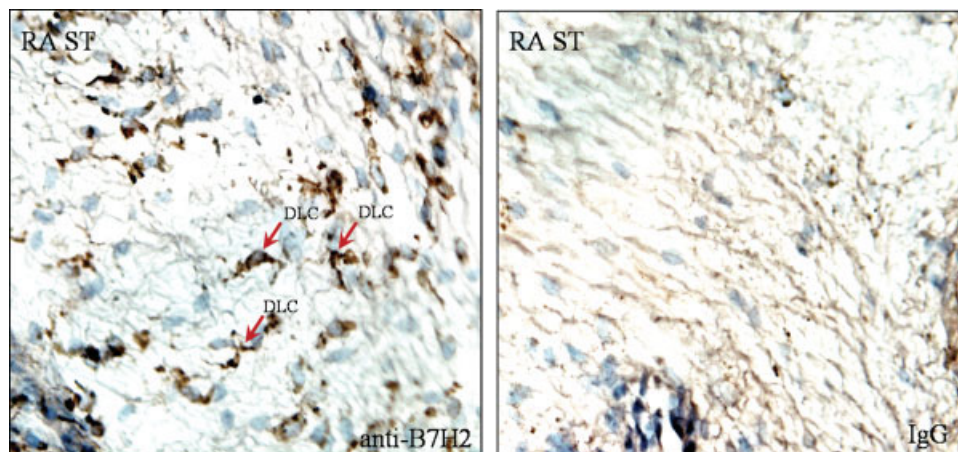


FIG. 8. Photomicrograph of RA ST immunostained for B7H2. As shown, intense B7H2 staining of ST APCs (dendritic-like cells) was found in RA compared to NL ST (indicated as DLC, see arrows; original magnification $\times 400$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1
B7 Molecule Staining in RA and NL Synovial Tissues

	Inflm score	Vasc (1-4)	Line %	Macs %	EC %	SM %	Lymph %	DC %	Fib %
NL ST									
B7RP.1									
Mean	1	2	38	1	0	0	0	11	0
SEM ±	0.0	0.0	21.2	0.8	0.0	0.0	0.0	6.7	0.0
No. of tissues	6	6	6	6	6	6	6	6	6
B7RP.2									
Mean	1	2	7	2	0	0	0	18	0
SEM ±	0.0	0.2	4.7	0.9	0.0	0.0	0.0	5.0	0.2
No. of tissues	6	6	6	6	6	6	6	6	6
B7H1									
Mean	1	2	0	0	0	0	0	12	0
SEM ±	0.2	0.2	0.0	0.2	0.0	0.0	0.0	7.2	0.0
No. of tissues	6	6	6	6	6	6	6	6	6
B7H2									
Mean	1	2	0	3	0	0	0	13	0
SEM ±	0.2	0.3	0.0	3.3	0.0	0.0	0.0	4.8	0.0
No. of tissues	6	6	6	6	6	6	6	6	6
RA ST									
B7RP.1									
Mean	2	2	36	6	0	0	0	16	1
SEM ±	0.3	0.3	20.3	3.0	0.2	0.3	0.0	7.0	0.9
No. of tissues	6	6	6	6	6	6	6	6	6
B7RP.2									
Mean	2	2	21	1	0	0	0	15	0
SEM ±	0.2	0.0	17.4	1.0	0.0	0.0	0.0	5.3	0.0
No. of tissues	6	6	6	6	6	6	6	6	6
B7H1									
Mean	3	3	15	5	0	0	0	6	0
SEM ±	0.3	0.3	12.2	4.1	0.0	0.0	0.0	1.4	0.0
No. of tissues	6	6	6	6	6	6	6	6	6
B7H2									
Mean	3	3	37	20	0	0	0	46*	1
SEM ±	0.2	0.2	20.7	14.1	0.0	0.0	0.0	14.2	1.0
No. of tissues	6	6	6	6	6	6	6	6	6

*P < 0.05 NL ST compared to RA ST.

Inflm score, inflammatory score; Vasc, vascular score; Line, lining cells; Macs, macrophages; EC, endothelial cells; SM, smooth muscle cells; Lymph, lymphocytes; DC, dendritic-like cells; Fib, fibroblasts.

before development of inflammation shows marked effects on EAE development (7). However, it is currently unknown whether these lymphocyte populations are specifically recruited to the RA joint by coexpression of chemokine receptors. We are currently examining these possibilities with in vitro multicolor flow cytometry experiments. [Nevertheless, leukocytes do over express ICOS and B7H2 in RA ST, as immunohistochemical analysis showed robust staining for lymphocyte ICOS and APC (dendritic like cell) B7H2 expression.] Interestingly, we identified B7 molecules on RA SF CD14+ monocytes known to down-regulate T-cell activation through PD-1 (B7H1 and 2), and upregulate T-cell activation through B7RP.1. [We also show in Table 1 that RA ST associated dendritic-like cells significantly express B7H2 compared to NL ST.] Thus, in the same joint, there appear to be both positive and negative influences, in which positive, pro-inflammatory forces may be dominating as the RA patient becomes symptomatic. It is also possible that negative feedback signals begin to dominate as joint inflammation resolves, for example, due to RA therapy. Thus, leukocytes bearing ICOS and B7 molecules may be critical mediators

of joint inflammation, partly responsible for the waxing and waning nature associated with RA.

Overall, these findings suggest that CD3+/CD4+ memory lymphocytes, because of upregulated costimulatory and ICOS molecule expression, are primed for lymphocyte recruitment and activation in RA. This data identifies increased populations of cell subsets expressing B7 molecules and ICOS in RA, and strongly indicates that ICOS and/or B7 molecule blockade may be a relevant target for RA treatment.

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